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Short Synopsis

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PRODUCTION AND PURIFICATION OF THERMOSTABLE XYLANASE

DEPARTMENT OF BIOTECHNOLOGY
FACULTY OF ENGINEERING & TECHNOLOGY

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ABSTRACT

A huge quantity of carbon in form of lignocellulosic waste is available from forests and agriculture. Lignocellulose or hemicellulose is a high molecular weight polymer of xylose and is a major component of cell walls of woody plants. Endo-1, 4 β-xylanase is an enzyme which plays a vital role in depolymerizing xylan which is major component of hemicelluloses. It is a well known fact that more than 40% of world's wood waste is burned, emitting gases and particles is responsible for immense pollution. This also results in complete destruction of valuable resource that is lignocelluloses which on the other hand, can be hydrolysed and used as a microbial media component for production of high value fermentation products. These xylanases catalyze the hydrolysis of xylan into sugars that can be utilized microbial cell growth and are efficiently produced by bacteria and fungi that thrive on degrading plant waste. For industrial applications it is important that xylanases are active at higher pH and possess thermostability at process temperature. Therefore, xylanases from bacteria are considered to be more suitable for industrial applications, than from fungi which have a low pH optima and thereby thermo labile. The present study is designed to screen for high xylanase yielding strains of thermophillic bacteria, optimization of xylanase production process, followed by purification and finally application of the purified xylanase for fermenting sugars. This would enable to develop a sustainable technology for production of high value commercial products from the waste lignocellulosic biomass.

Keywords: Hemicelluloses Xylanase, fermentation, xylanase purification

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Introduction

Xylan is a major component of hemicelluloses, present in cell walls of woody plants and abundantly available in organic wastes from forest, and agricultural residues. Xylanases are group of enzymes that depolymerize xylan molecules into xylose. They are gaining worldwide attention as they enable the use of available agro-wastes for synthesis of various industrially important chemicals. The potential biotechnological applications of xylanase include food and feed industry, textile industry, bio-fuel production, paper and pulp industries and in waste treatment.

A group of xylanases can be used to degrade the waste lignocellulosic biomass to fermentable sugars which can be used as carbon source in microbial medium. Many a times, the major impediment in the commercial viability of a bioprocess, is the high cost of production media, especially the sugar component, that acts as carbon source for the growth of process organism. One of the other prominent applications of xylanase is in the paper and the pulp industry for bleaching of kraft pulp. In the conventional paper making processes, manufacturers use many hazardous chemicals that are polluting the environment. Owing to environment protection laws, there is a need to look for a viable alternative bioprocess. It has been established, that xylanase treatment of kraft pulp facilitates the extraction of lignin from pulp and leads to a significant reduction in use of chlorine dioxide (ClO_2), a hazardous chemical required to bleach the paper pulp (Azeri *et al*, 2010).

Xylanases are hydrolytic enzymes that randomly cleave the β-1, 4 backbone of the complex plant cell wall polysaccharide ie: xylan. However, complete enzymatic hydrolysis of xylan into its constituent monosaccharides requires the synergistic action of a consortium of xylanolytic degrading enzymes. This is due to the fact, that xylans from different sources exhibit a significant variation in composition and structure. The most important enzyme is endo-1, 4-xylanase, which initiates the conversion of xylan into xylooligosachharides. Xylosidases or debranching enzymes (L-arabinosefuranosidase and glucuronidase) and esterases (acetyl xylan esterase, feruloyl esterase) allow the complete degradation of the xylooligosachharides to their monomeric constituents.

Many microorganisms including bacteria (Kamble and Jadhav, 2012), fungi (Tallapragada and Venkatesh, 2011) and yeast (Otero *et al,* 2015) have been reported to produce xylanases. The properties of xylanases obtained from different microorganisms differ in their activity, stability and specificity. The ideal candidate for xylanase, for different applications should be

thermostable, alkali tolerant and stable. Moreover, xylanases used for biobleaching of paper pulp should be free from cellulase activity otherwise it would damage the paper (Subramaniyan and Prema, 2002).

Potential application of xylanases in biotechnology include bio-bleaching of wood pulp, treating animal feed to increase digestibility, processing food to increase clarification and converting lignocellulosic substances to feedstock and fuels. Bioconversion of lignocelluloses to fermentable sugars has huge economic prospect. It is because massive accumulation of agricultural, forestry and municipal solid waste residues create large volume of low value feedstock. Xylanolytic system comprising of different types of enzymes such as xylanases, β-xylosidases, and the various debranching enzymes would ensure degradation of low value feed stock into the utilizable sugars. Production of environmentally friendly fuel is gaining great importance as the energy sources are shrinking. Several researchers have reported the use of xylanases for pretreatment of lignocellulosic mass to produce fermentable sugars and further processing for production of ethanol as a biofuel (Shilpa C. *et al*, 2013).

In the present work, the objective is to isolate a novel thermophillic bacterium that possesses capability of xylanase production. A thermostable and alkali stable xylanase would be produced using a suitably optimized media for maximization of production. A cost effective and industrially relevant purification strategy would be adopted to obtain xylanase with high specific activity. The application of this purified xylanase for production of fermentable sugars and subsequent fermentation into high value products would be optimized. A technology enabling production, purification and application of thermostable xylanase would be developed in the proposed work.

Literature review

Microbial xylanases are used in many industries such as food and feed, textile, paper etc. It is also used in treatment of waste. Moreover, many new applications are emerging, due to the global thrust for sustainable technologies. Already established uses are being tested for better economic viability. This reflects that there is a wide scope for research on maximizing the production of xylanases which are also cost effective.

Xylanases are known to be single chain glycoproteins having molecular weight ranging from 6 kDa to 80 kDa, active between pH 4.5-6.5 and at temperature between 40^{o}C and 60^{o}C (Butt *et al*, 2008). The complete amino acid sequence of endoxylanases was obtained from *B. subtilis*

(Khandeparker *et al*, 2011). It was reported that *B. subtilis* endoxylanase is composed of 185 amino acid residues. The partial amino acid sequences have also been reported for two endoxylanases. The first 72 amino acid residues from the N-terminal were sequenced for an endoxylanases of *Cryptococcus albidus* (Morosoli *et al*, 1986), while the first 27 residues from the N-terminal were sequenced for endoxylanase of *Schizophyllum commune* (Paice *et al*, 1978).

Xylanases have been reported to be very widely distributed in nature (Otero *et al*, 2015; Kamble and Jadhav, 2012). The microorganisms elaborating xylanases activity do so to obtain easily utilizable carbon sources such as xylose and its oligosaccharides, whereas plant pathogenic species use xylanases to attack plant cells in order to facilitate their entry and bring about colonization. Xylanases are also produced by higher eukaryotes, including protozoa, insects, and snails and germinating plant seeds (Taiz and Honigman, 1976). The intestines of ruminants are colonized with many microorganisms elaborating xylanases activity which help in digestion of hemicellulosic component of feed (Dekker and Richards 1976). Amongst the prokaryotes, bacteria and cyanobacteria from marine environments produce xylanases (Dekker 1985).

Researchers have attempted production of xylanase using different species of bacteria and fungi. A new species of thermo-alkalophillic *Bacillus*, that was found to be similar to *Bacillus arseniciselenatis* DSM 15340, produced extracellular xylanase under solid state fermentation when wheat bran was used as carbon source (Kamble and Jadhav 2012). A new strain of *Bacillus*, which was able to produce extracellular alkaline thermostable xylanase was isolated and characterized (Hiremath *et al*, 2011). Ten thermophillic microbes were isolated from degraded wood; cow feed sample, sugarcane bagasse and soil sample on PDA plates by Kumar *et al*, 2010. Xylanase activity was detected from a strain of *Bacillus cereus* (Roy and Habib, 2009), which is rare. *Bacillus pumilus* MTCC 8964 showed significant amount of xylanase production using selective growth and nutrient conditions. Different agro-residues, as substrates, in solid state fermentation were evaluated for xylanase production by a thermo-tolerant *Bacillus* isolate (Gupta and Kar, 2008). Corn cob, an abundantly available agro-residue in India was successfully used as a solid state support by *B. licheniformis* isolate for xylanase production. Prominently thermostable activity of xylanase has been derived from bacteria belonging to *Bacillus sp.* A thermophillic

bacterium that produces both xylanase and β-xylosidase has been isolated (Nanmori *et al*, 1990) and was identified as *Bacillus stearothermophilus.*

A novel thermo-alkalophillic xylanase was isolated from *Enterobacter* spp. with significant activity grown on basal salt solution using birchwood xylan as a substrate (Sharma *et al*, 2009). Following phyla have been identified for xylanolytic activity 16S rRNA analysis: *Acidobacteria, Actinobacteria, Bacteroidtes, Firmcutes, Proteobacteria Verrucomicrobia* (Pereira *et al*, 2006). Effect of some cultural conditions on the xylanase enzyme production by *Streptomyces sp*. (strain lb 24D) and to investigate its potential to produce xylanase utilizing tomato pomace as a substrate has also been achieved (Rawashdeh *et al*, 2005). The xylanase producing bacterial strains were also isolated from drain water (Roy and Abedin, 2002) and were identified as *Aeromonas sp.*

Xylanases are also produced by higher eukaryotes, including protozoa, insects, and snails and germinating plant seeds (Taiz and Honigman, 1976). Amongst the prokaryotes, bacteria and cyanobacteria from marine environments produce xylanases (Dekker 1985).

Table 1. Important bacterial species showing xylanase production and their characteristics

SN	**Bacterium**	**Carbon Source used in production medium**	**Reference**
1	*Bacillus pumilus*	Maltose	Kumar *et al*, 2010
2	*Bacillus cereus*	Oat Xylan	Roy and Habib 2009
3	*Enterobacter* spp	Birchwood xylan	Sharma *et al*, 2009
4	*B.lichniformis*	Corn	Gupta and Kar 2008
5	*Streptomyces sp*.	Glucose, Xylose, Xylan, Arabinoxylan, Tomato pomace	Rawashdeh *et al*, 2005
6	*Aeromonas sp*	Oat Xylan	Roy & Abedin 2002
7	*Bacillus stearothermophilus*	Oat Xylan	Nanmori *et al*, 1990

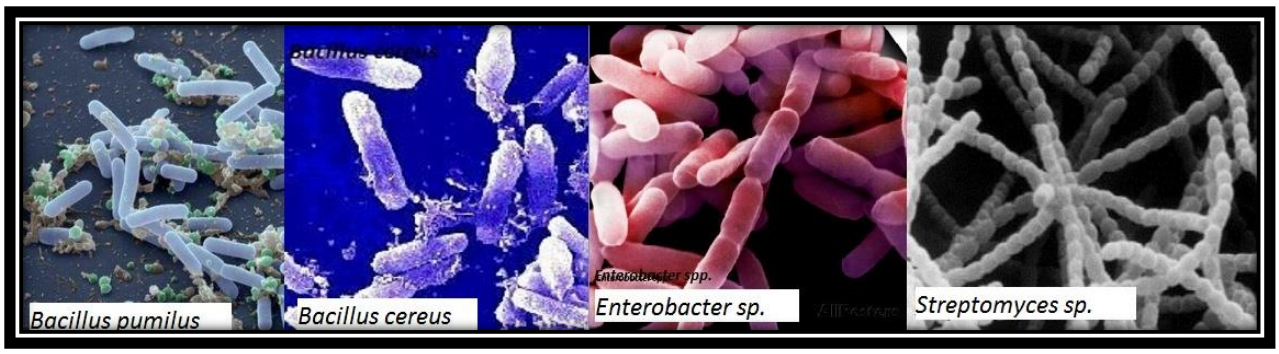

Figure 1: Various bacterial species which show xylanase production

Xylanases have been isolated majorly from fungal species namely *Aspergillus, Trichoderma, Humicola and Penicillium*. In a study, 54 different endophytic fungi were isolated; out of which 30 showed xylanolytic activity (Wipusaree *et al*, 2011). After screening of xylan liquid culture, isolate that yielded the highest xylanase production, was selected for further evaluation. The isolate was found to be endophytic fungus *Alternaria alternata*.

Xylanase production by wild type *Aspergillus niger* ANL301, newly isolated from wood-waste, was monitored at 24 h intervals for a period 168h in media containing different carbon sources. The carbon sources were oat-spelt xylan and three agro wastes - sawdust, sugarcane pulp and wheat bran (Okafor *et al*, 2007). Out of all the agro wastes, wheat bran was considered most reliable source of xylanase production and economical. Waste lignocellulosic material (orange, pomace, orange peel, lemon pomace, lemon peel, apple pomace, pear peel, banana peel, melon peel and hazelnut shell) were used as substitutent of peptone in the medium for the production of xylanase from *Trichoderma harzanium* 1073 D3. It was observed, that use of melon peel followed by apple pomace and hazelnut shell maximum activity resulted in maximum xylanase activity (Seyis and Aksoz, 2005).

Besides fungi and bacteria a few species of yeast, such as *Cryptococcus albidus* (Biely *et al*, 1981); *Aureobasidium pullulans* (Leathers 1988) and *Cryptococcus laurentii* (Otero *et al*, 2015) have also been reported to produce extracellular endoxylanase.

Table – 2 Important fungal species exhibiting xylanase production

SN	Fungus	Carbon Source used in production medium	Reference
1	*Humicola Ianuginosa*	Wheat bran	Irshad *et al*, 2011
2	*Penicillum janthinellum*	Oat Xylan	Abirami *et al*, 2011
3	*Trichoderma reesei*	Birchwood Xylan and Oat Xylan	He *et al*, 2009
4	*Aspergillus niger*	Wheat bran	Okafor *et al*, 2007
5	*Aspergillus terreus.*	Glucose	Pal *et al*, 2006
6	*Trichoderma harizanium* Rifai	Vegetable waste	Seyis and Aksoz 2005
7	*Trichoderma longibrachiatum*	Glucose	Royer and Nakas 1990

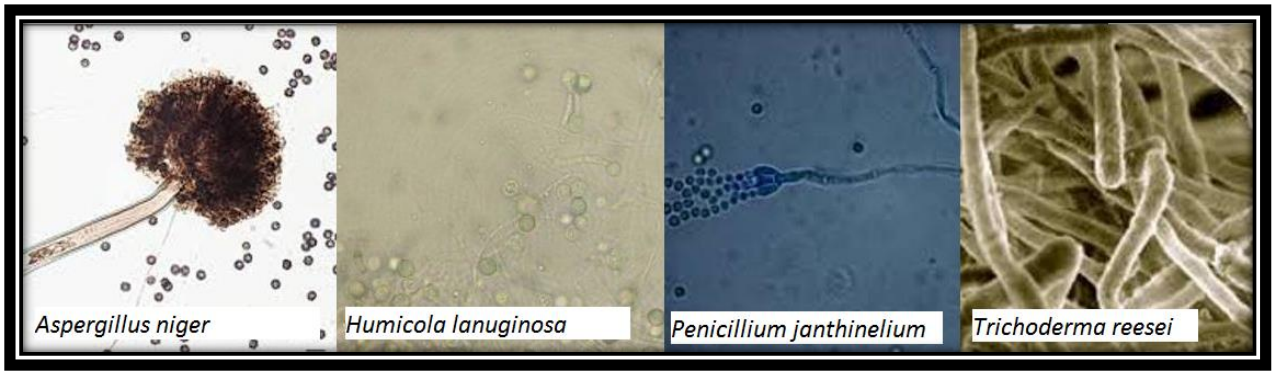

Figure 2: Various fungal species which show xylanase production

With the recent developments in genetic engineering, enzyme xylanase can be modified specifically to suit its different industrial applications. The kinetics of growth and product formation was studied in recombinant *E. coli* DH5α pTP510. It was observed that xylanase production was growth associated process and the enzyme secretion was greatly dependent on cell concentration and the specific growth rate of recombinant *E. coli* DH5α pTP510 (Rusli *et al,* 2009). Xylanase production by genetically engineered nonpathogenic and non-toxigenic strains of *Bacillus subtilis* was studied (Olempska-Beer 2004). Using appropriate transformation vector, three different strains of *B.subtilis* were created. Out of these three strains, two were found to

express wild type xylanase whereas third strain expresses xylanase that was resistant to the xylanase inhibitor present in wheat. An investigation was conducted to determine the optimal culture conditions for enhanced biosynthesis of xylanase by a chemically mutated strain of *Aspergillus niger* GCBCX-20 (Haq *et al*, 2004). It was observed that enzyme production was greatly reduced if pH of the medium was more than 5.0. Degradation of xylan to D-xylose by recombinant *Sccharomyces cerevisiae* co-expressing the *Aspergillus niger* β-Xylosidase (xlnD) and the *Trichoderma reesei* Xylanase II (xyn2) Genes was carried out (Grange *et al*, 2001) for complete xylan hydrolysis. Newly formed recombinant β-xylosidase was found to possess maximum activity at 60°C and pH 3.2. However, these technologies are still in their nascent state. ***Much work has to be carried out to transfer the technology from laboratory to industry.***

Xylanases have been purified using different types of purification strategies (Ratanachomsri *et al*, 2006). The unit operations used for concentration, such as ultra filtration, anion exchange chromatography and gel filtration have been employed for purification of xylanase. In a study, molecular mass of partially purified enzyme was found to be very low with solvent precipitation (Kumar *et al*, 2010). High performance liquid chromatography (HPLC) has also been used (Kluepfel *et al*, 1990) for purification. Extracellular xylanase free of cellulose, produced by the alkalophilic bacteria *Bacillus pumilus* was purified by precipitating with ammonium sulfate, Q-Sepharose chromatography.

Xylanase is being used in bleaching of kraft pulp has an advantage of decreasing the use of chlorine, production of chlorinated organic chemicals and increasing the brightness of paper. Xylan is hydrolyzed and lignin is released by enzymatic treatment of the pulp. Hence chlorine based bleaching chemicals that are polluting the environment are not needed (Kenealy and Jeffries, 2003). Chemical bleaching procedures are damaging to the environment, because it releases numerous toxic chlorinated organic substances such as chloro-phenols, in the environment. Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), toxicity, mutagenesis and carcinogenic and bioaccumulative properties increase with the production of organo-chlorides from paper mills (Bajpai 1999). In hope of reducing capital and operating costs and minimizing its environmental impact, the paper industry is searching for biological agents instead of chemicals used in the paper making process.

In cereals, like barley, polysaccharides such as arabinoxylans are present constituting about 4-8% of barley kernel. They also represent about 25% and 70 % of the cell wall polysaccharides of endosperm and aleurone layer respectively. The arabinoxylans are partly water soluble and result in a highly viscous aqueous solution. This high viscosity of cereal grain water extract leads to problems such as decreased rate of filtration or haze formation in brewing processes. High viscosity is a negative parameter for the use of cereal grains in animal feeding. A better solution for this problem could be derived from the application of xylanases for pre-treating the arabinoxylan containing substrates. The xylanolytic enzymes are also employed for clarifying juices and wines, for extracting coffee, plant oils and starches, for improving the nutritional properties of agricultural silage and grain feed. Xylanases also find application in rye baking where the addition of xylanase makes the doughs soft and slack. Xylanases are used as dough strengtheners since they provide excellent tolerance to the dough towards variations in processing parameters and in flour quality. They also significantly increase volume of the baked bread.

Description of Topic

Xylanase has a huge potential to be used in different process industries, importantly in food & feed, biofuels, fertile, paper & pulp. Most of these applications require a xylanase that could sustain the higher temperatures and pH. Therefore, to meet the demand of industry, a technology for production and purification of thermostable xylanase would be developed. In the present study, the strains would be selected on the basis of higher xylanase productivity. The process parameters such as pH, temperature, rate of agitation would be optimized for maximization of xylanase production. Thermo stable xylanolytic micro organisms thrive in many natural niches such as soils containing high amount of decaying plant matter, marshy areas and water bodies. The isolation and screening would be carried out using enrichment media and hyper producers of xylanases would be selected. The yield of xylanase would be enhanced by optimization of upstream process. The crude xylanase obtained in the fermentation broth would be subjected to step by step purification techniques. The purified xylanase would be applied for degradation of lignocellulosic biomass and degradation would be analyzed. The fermentable sugars so formed during lignocellulosic degradation would be used as carbon source for production of bioethanol.

Objectives of the study

1. To screen bacterial strains from various sources for thermostable xylanase production.
2. To obtain pure culture of bacterial strain that shows thermostable xylanase production.
3. To characterize the selected strain by microscopic and biochemical methods.
4. To optimize Xylanase production in shake flask cultures.
5. To perform purification and characterization of xylanase.
6. To study bioethanol production using xylanase treated lignocellulosic waste.

Methodology to be adopted

- **Collection of samples:** Soil and water samples would be collected from Damdama Lake, Sohna and Shiv kund (hot water geyser), Sohna, Haryana and their surroundings for the study of enzyme producing microorganisms. The water and soil samples would be collected from areas having decaying plant matter.
- **Screening and Issolation of xylanase producing bacteria**: The collected samples would be screened on enrichment media containing xylan as the sole carbon source. The isolates would be visualized by Congo red dye binding method (Teather and Woods, 1982).
- **Microscopic and biochemical characterization of selected strains:** The microscopic and biochemical characterization of the selected strains would be carried out using standard microbial techniques as given in the Bergeys Manual (Buchanan and Gibbons, 1974).
- **Production process optimization:** The production medium would be optimized with respect to important constituents such as carbon and nitrogen sources. The process parameters such as temperature, pH, rate of agitation and other micro-constituents would be optimized in order to enhance xylanase production.

Assay of Xylanases

The substrate used for this assay is birchwood xylan (Ghose and Bisaria 1987). Xylanase activity would be assayed by measuring the increase in reducing end-groups by dinitrosalicylic acid (Miller 1959).

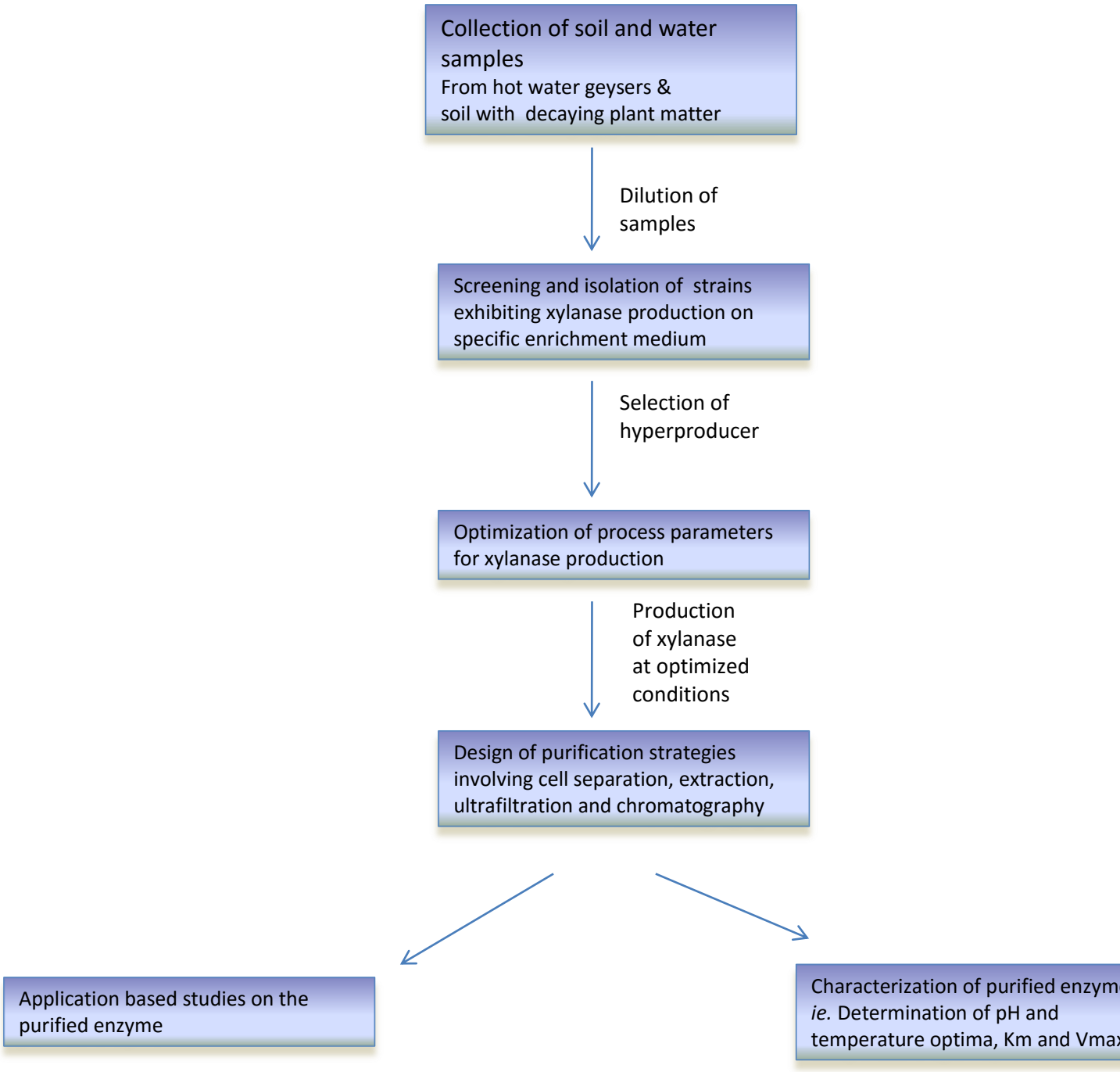
Collection of soil and water samples
From hot water geysers & soil with decaying plant matter
Dilution of samples
Screening and isolation of strains exhibiting xylanase production on specific enrichment medium
Selection of hyperproducer
Optimization of process parameters for xylanase production
Production of xylanase at optimized conditions
Design of purification strategies involving cell separation, extraction, ultrafiltration and chromatography
Application based studies on the purified enzyme
Characterization of purified enzym
ie. Determination of pH and temperature optima, Km and Vmax

Figure 3: Schematic depicting plan of research work

Proposed Outcome

Xylanase is an important process enzyme owing to its application in industries such as paper, food and feed, textiles etc. It is emerging as a vital enzyme for sustainable waste management and production of biofuel from lignocellulosic waste. Market demand of xylanases has increased significantly during last few decades. However, the only bottleneck in the application is the higher cost of production. In most of the processes the cost of the medium is due to high cost of carbon source. In the present project, the objective is to develop a cost effective xylanase production and purification process. The purified enzyme would be characterized and studies on application of this purified enzyme for lignocellulosic degradation would be carried out. A hyper producer bacterial strain would be isolated and a high yield economical production technology will be developed for xylanase. A purification strategy would be designed that would be viable for scale up as an industrial enzyme.

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